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TITLE: Resistance to Tamoxifen: A Consequence of Altered p27Kip1 Regulation During Breast Cancer Progression

PRINCIPAL INVESTIGATOR: Joyce M. Slingerland, M.D.,Ph.D.

CONTRACTING ORGANIZATION: Sunnybrook Health Science Centre
Toronto, Ontario, M4N 3M5, Canada

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Joyce M. Slingerland, M.D.,Ph.D.

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13. ABSTRACT *(Maximum 200 words)*

This grant addresses the molecular mechanisms of resistance to the anti-estrogen, Tamoxifen, in breast cancers. Both estrogens and antiestrogens influence the cell cycle during the G1 phase. In breast tumors, loss of the cdk inhibitor, p27, is associated with a poor prognosis and with steroid resistance. We postulate that altered p27 degradation in breast cancer may contribute to resistance to antiestrogen therapy. In the last year, we have assayed cell cycle effects of estradiol in the ER+ breast cancer cell line, MCF-7. MCF-7arrests in G1 upon removal of estradiol or on addition of Tamoxifen. Upon re-addition of estradiol, cells re-enter the cell cycle with onset of S phase by 12 hours and loss of both p21 and p27 proteins. Cyclin D1 associated kinase activity rose transiently in early G1. Activation of cyclin E/cdk2 kinase in mid-late G1 was accompanied by loss of cyclin E-associated p21 and p27. The requirement for p27 in the quiescence induced by estradiol depletion was investigated using antisense p27 oligonucleotides. Introduction of the antisense oligonucleotides into quiescent estradiol depleted MCF-7 cells reduced p27 levels five fold. Despite the continued absence of estradiol, the p27 antisense treated group entered into S phase, with an S phase fraction of 28 % at 34 hours post-transfection, while control cells remained arrested. Thus, the loss of p27 was sufficient to mimic the effect of estradiol on G1-to-S phase progression in MCF-7 cells. These data suggest that loss of p27 is critical for estradiol dependant stimulation of breast cancer proliferation and an increase in p27 is essential for cell cycle arrest following an inhibition of estradiol signaling. Ongoing studies address how these effects are altered in steroid resistant breast cancer.

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ANNUAL REPORT

US Army Medical research Command Award #DAMD17-98-1-8158 and #DAMD17-98-1-8159

Grant Title: Resistance to Tamoxifen: a consequence of altered $p27^{Kip1}$ regulation during breast cancer progression

PI: J.M. Slingerland

INTRODUCTION

While approximately 70% of breast cancers express the estrogen receptor (ER) at diagnosis, only two thirds of these will respond to antiestrogens such as Tamoxifen (TAM). Unfortunately, ER positive tumors that are initially responsive, invariably acquire resistance to hormonal therapies during disease progression (reviewed in (1)). TAM-resistant tumors usually show continued expression of the ER (2; 3). Estradiol, in association with other steroids and growth factors, regulates cell proliferation and development in the mammary gland. However, the mechanisms whereby estradiol mediates proliferation are unclear. Thus, the elucidation of mechanisms whereby estradiol:ER influences cell cycle regulators and how these are blocked by Tamoxifen is highly relevant to the development of new treatments for steroid resistant breast cancer. Both estrogens and antiestrogens influence cell cycle regulators during the early-to-mid G1 phase of the cell cycle (4). The cell cycle is governed by a family of cyclin dependent kinases (cdks), whose activity is regulated by binding of positive effectors, the cyclins, by phosphorylation and by negative regulators, the cdk inhibitors (reviewed in (5-7)). Our immunohistochemical analysis of the cdk inhibitor, p27or kinase inhibitor protein 1 (KIP1), in primary breast tumors showed strong p27 staining in normal mammary epithelial cells and suggests a role for this cdk inhibitor in maintaining mammary cell quiescence (8). That p27 protein levels are frequently reduced in primary breast cancers and this correlates with poor prognosis, further suggests that p27 is an important negative regulator of the normal breast cell cycle (8-10) . The cellular abundance of p27 is importantly regulated by ubiquitin-mediated proteolysis (11). p27 protein levels decrease when quiescent MCF-7 breast cancer cells are stimulated to reenter the cell cycle with estradiol treatment and p27 increases when antiestrogens induce G1 arrest (12; 13) and Slingerland and Cariou, manuscript in prep.). The hypothesis underlying this proposal is that estrogen stimulates breast cancer cells to enter the cell cycle by activating p27 phosphorylation, thereby signaling its degradation and antiestrogens act to block p27 phosphorylation leading thereby to p27 accumulation and G1 arrest. Altered p27 degradation in breast cancer cells may underlie resistance to cytostasis by Tamoxifen. Using a model of ER positive, estradiol-sensitive breast cancer lines and steroid resistant derivative sister cell lines, work proposed aimed to determine how estrogen:ER regulates p27 and other effectors of cell cycle progression and how these mechanisms are altered during breast tumor progression to a steroid resistant state. These studies aim to increase our understanding of the estrogen-autonomous growth that is seen in both de novo and secondary hormone resistant advanced breast cancers. A better understanding of how cell cycle regulators, particularly the cdk inhibitor p27, are altered during progression to steroid-resistance may ultimately lead to the generation of novel therapeutic strategies for breast cancer.

HYPOTHESES AND SPECIFIC AIMS

Hypothesis: When this grant was first submitted, we proposed the following **hypothesis** to be pursued by **3 specific aims**. We originally postulated that estrogen stimulates breast cancer cells to enter the cell cycle by activating p27 phosphorylation, thereby signaling its degradation. We also postulated that antiestrogens act to block p27 phosphorylation leading thereby to p27 accumulation and G1 arrest. Altered p27 degradation in breast cancer cells may underlie resistance to cytostasis by Tamoxifen. This hypothesis was to be pursued in the following Specific Aims.

AIM 1. We will compare effects of antiestrogen and estrogen on steroid sensitive and insensitive breast cancer lines which express the ER. We will test **a)** whether phosphorylation of p27 precedes the reduction in p27 levels following estradiol stimulation of steroid sensitive breast cancer lines; **b)** whether this is blocked by antiestrogens; and **c)** how estradiol sensitive and resistant lines differ in p27 protein expression, stability, p27 phosphorylation, localization and binding of novel p27-associated proteins.

AIM 2. To test whether increased ubiquitin proteasome activity lowers p27 levels in estradiol stimulated MCF-7 cells, we will use chemical proteosome inhibitors following estradiol stimulation of MCF-7; we will also use a temperature sensitive (ts) mutant of the ubiquitin activating enzyme E1 to determine how loss of this activity affects p27 levels, p27 phosphorylation and entrance into the cell cycle.

AIM 3. We will determine whether p27 is critical to growth inhibition by antiestrogens by introducing inducible antisense p27 into MCF-7 or by using antisense p27 oligonucleotides.

The original statement of work, SOW (proposed June 1997) and the revised SOW approved May 1998 are appended. I will refer to both the work of the Specific Aims proposed above and the SOW throughout the Progress Report as appropriate.

SUMMARY OF PROGRESS ON GRANT

A) Summary of Results from Manuscript in Preparation

p27 is essential for cell cycle arrest following interruption of estradiol signaling in MCF-7 breast cancer cells. S. Carious, J. Donovan, N. Bhattacharya and J. Slingerland, in preparation for PNAS

A large part of the work of specific aim 1 has been completed in the last year. We have examined the effects of estradiol and antiestrogens on cell cycle regulators, including p27, in the MCF-7 line. The work completed which is described below is essential groundwork for some of the studies which remain to be carried out in TASK 1 of the revised SOW (formerly Task 2). Review of the summary of results below will also show that a significant part of the work of TASK 2 (formerly Task3) has been completed.

Cell Synchronization

Asynchronously growing MCF-7 cells were rendered quiescent (2% S phase) by depletion of estradiol by transfer to medium supplemented with charcoal stripped serum. Cells were released from quiescence by re-addition of estradiol 10^{-8}M. At intervals following the addition of estradiol,

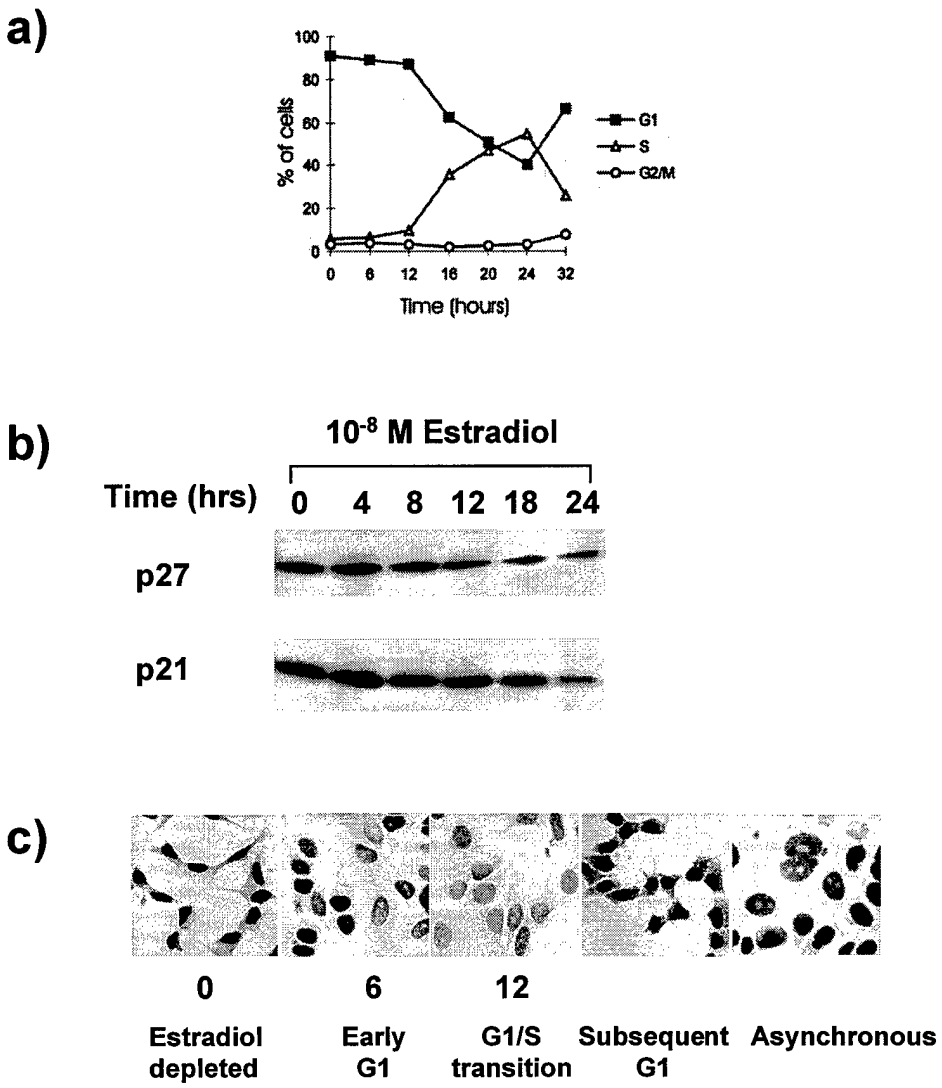


FIG. 1 Estradiol stimulates quiescent MCF-7 to re-enter the cell cycle. *(a)* Cell cycle distribution graphed from dual BrdU/PI staining. After 48 hours of steroid depletion, 10^{-8} M estradiol was added to the culture media and cells were recovered for flow cytometric analysis at the times indicated. Cells re-enter the cell cycle approaching the G1/S phase transition at 12 hours. *(b)* Western blot analysis of cdk inhibitors shows a decrease in both p21 and p27 as cell progress through G1 phase. *(c)* Immunohistochemical analysis of p27 across the cell cycle. Cells were plated onto glass slides, synchronized by estradiol depletion and stimulated by repletion of estradiol as in *(a),* above. p27 was stained as described in Materials and Methods, at intervals following re-addition of estradiol: at times 0 hrs (G0), 6 hrs (early G1), 12 hrs (G1/S) and 36 (subsequent G1). p27 staining of asynchronously growing MCF-7 cells is shown in the far right panel.

cells were harvested for flow cytometry to analyze the cell cycle profile and for extraction of protein. As shown if Fig 1a, MCF-7 cells progressed synchronously into G1 and approached the G1-to-S phase transition within 12 hours following re-addition of estradiol. Peak S phase entrance was observed at 24 hours (55% S phase cells).

p21 and p27 protein levels fall following estradiol stimulation of MCF-7 cells

Cell cycle regulator protein levels were assayed at intervals as MCF-7 cells moved from G0 into S phase. Levels of cyclin E, cdk2, cdk4, and cdk6 remained constant. $p15^{INK4B}$ protein levels fell as cells moved into G1. Cyclin D1 was not detected in G0 but rose early in G1. Cyclin A protein rose as cells entered S phase (not shown). Both p21 and p27 levels fell significantly. The loss of p27 was more notable and preceded that of p21 (Fig 1b). Immunohistochemical analysis of p27 showed variable levels from strong nuclear p27 staining to complete absence of protein in asynchronously growing MCF-7 (right-hand panel, Fig 1c). Estradiol depleted cells, at time 0 hrs, showed the highest levels of exclusively nuclear p27. Nuclear p27 staining was reduced in G1 (T=6 hrs) and fell to a nadir at 12 hours as S phase entrance began. Re-accumulation of p27 accompanied entrance into the subsequent G1.

Increased binding of p21 and p27 accompanies cyclin D-associated kinase activation

Cdk 4 complexes were assayed at intervals following estradiol stimulation of quiescent MCF-7. While cdk4 levels were constant, the amount of cdk4-bound cyclin D1 increased to a maximum at 6-9 hours following addition of estradiol. The assembly of cyclin D1/cdk4 complexes was accompanied by increased association of both p21 and p27 (Fig 2. The profile of the assembly of cyclin D1-binding to cdk4 paralleled the profile of cyclin D1-associated kinase activation, with peak activation detected by 6 hours.

Loss of cyclin E-associated p27 accompanies activation of this kinase

Activation of cyclin E-dependent kinase was detectable by 8 hours following release from G0 and was associated with losses of p27 and to a lesser extent p21, from cyclin E/cdk2 complexes (Figs 2 c and d). Activation of cyclin E dependent kinase was also accompanied by an increase in the proportion of threonine-160 phosphorylated cdk2 bound to cyclin E (as shown by the shift from the slower to faster mobility form of cyclin E-bound cdk2).

ER-blocking drugs increase cyclin E-associated p27 and induce G1 arrest

Asynchronously growing MCF-7 cells were treated with either Tamoxifen, or the estrogen receptor blocking drug, ICI^{182780} or transferred to medium supplemented with charcoal-stripped estradiol-depleted serum. In all cases, cells showed a progressive increase in the proportion of cells in G1 and a loss of cells from S phase over the subsequent 48 hours (data shown for ICI^{182780}, Fig 3a and b). Kinase inhibitor protein (KIP) levels increased following each of these forms of interruption of estradiol signaling (TAM, ICI^{182780}, and estradiol depletion), with p27 levels rising by 5 fold over 48 hrs. The inhibition of cyclin E-cdk2 activity was associated with a dramatic rise in cyclin E-associated p27 (Fig3b).

Antisense p27 simulates quiescent, estradiol-depleted cells to exit G0 and progress into S phase

The data presented in Figs 1 to 3 suggest that changes in p27 may play a key roles in the activation of cell cycle progression by estradiol and in the inhibition of G1 progression that occurs following

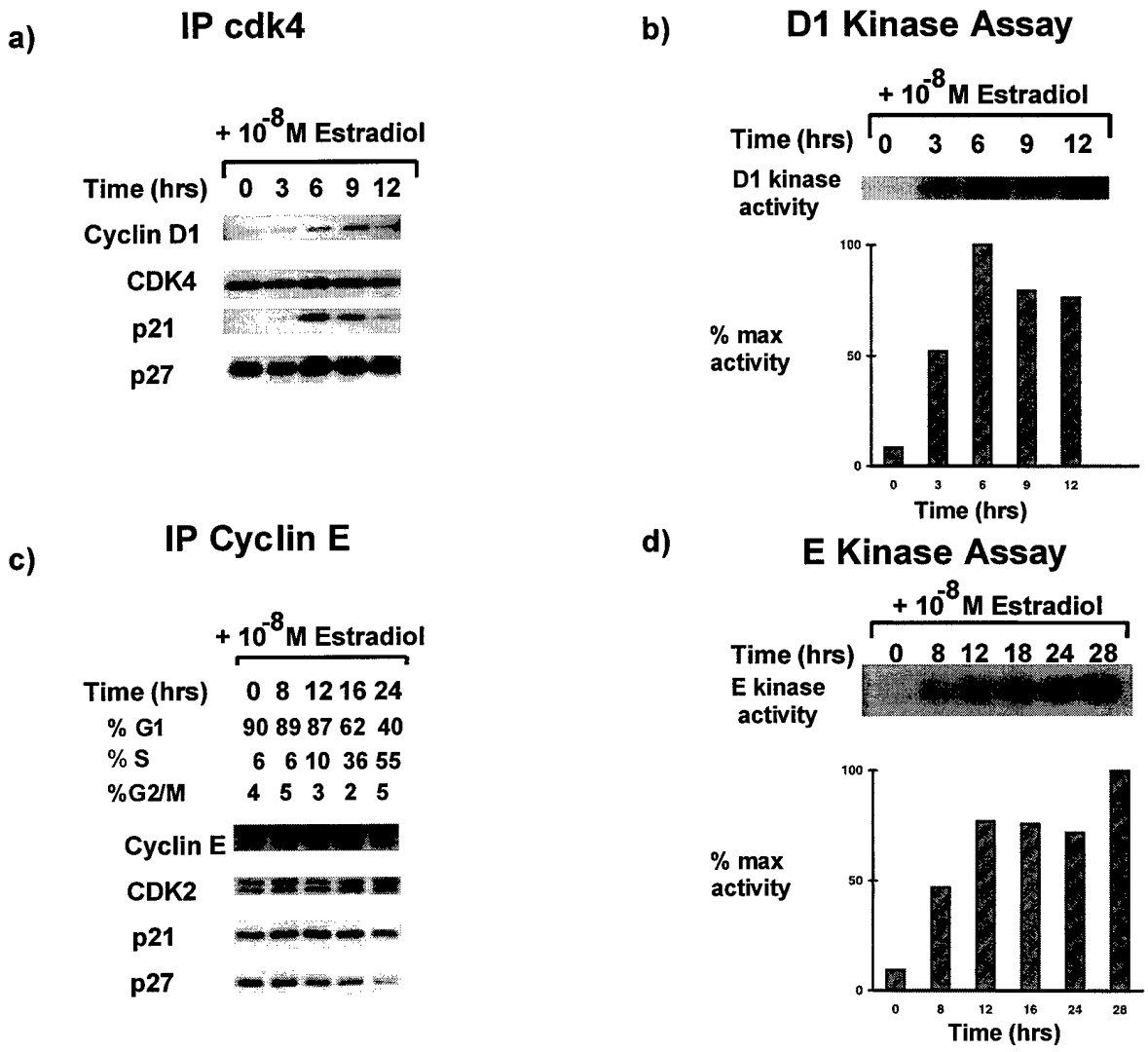
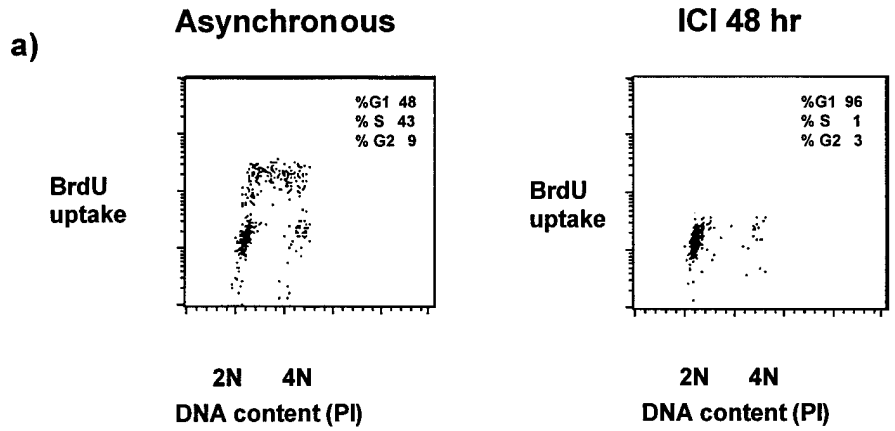


FIG. 2 G1 cyclin-dependent kinase complexes and activities. MCF-7 cells were synchronized by steroid depletion as in FIG 1 above, and released into the cell cycle by addition of 10^{-8} M estradiol to the culture medium. Lysates were recovered at the indicated times thereafter. *(a)* Cdk4 complexes were immunoprecipitated, resolved, and associated proteins detected by Western blotting. In response to estradiol, the level of immunoprecipitated cyclin D1 in cdk4 complexes increased in early G1. Cdk4 associated p21 and p27 were increased between 3 and 6 hrs *(b)* Immunoprecipitable cyclin D1-associated GST-pRb kinase activity peaked in early G1. *(c)* Cyclin E complexes were immunoprecipitated and associated proteins detected with the indicated antibodies. While cyclin E levels remained constant, levels of associated p27 and later p21 fell and cyclin E-associated cdk2 became more thr160 phosphorylated (shift to faster mobility) as cells progressed from G0 to S phase. *(d)* Immunoprecipitable cyclin E-associated histone H1 kinase activity increased as cells approached S phase.



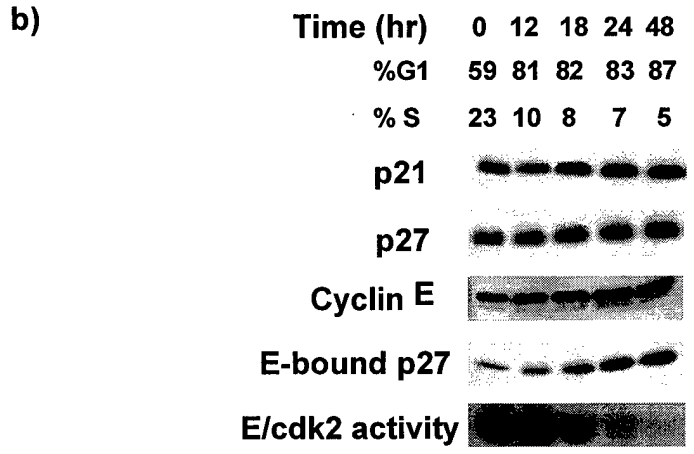


FIG. 3 The effects of antiestrogens on the cell cycle. *(a)* The cell cycle profiles of asynchronously proliferating MCF-7 and cells treated for 48 hrs with the antiestrogen, ICI^{182780} (10^{-5}M) are shown. Cells were pulse labeled with BrdU and then stained with antiBrdU-conjugated FITC and counterstained with propidium iodide (PI) for flow cytometric analysis. DNA content is on the X axis and BrdU uptake on the Y axis of the graphs. *(b)* At the indicated times following addition of the antiestrogen, cells were recovered for flow cytometry and for protein analysis. Both p21 and p27 levels rose over the 48 hr time course. The levels of cyclin E-bound p27 rose in association with loss of cyclin E-dependent kinase activity.

blockade of the estrogen receptor. The addition of estradiol to estradiol-depleted, quiescent cells stimulated exit from G0 and progression through G1 into S phase. The activation of cyclin E-cdk2 was associated with loss of p27 from these complexes. Similarly, interruption of estradiol signaling by the addition of ER blocking drugs or by removal of estradiol lead to a dramatic increase in cyclin E-associated p27, loss of cyclin E-dependent kinase activity and G1 arrest. While these data support the notion that the increased binding of p27 to cyclin E/cdk2 is critical to the G1 arrest accompanying loss of estrogen signaling, they are merely correlative. Thus we addressed the requirement for p27 in the quiescence induced by estradiol depletion using antisense p27 oligonucleotides.

Asynchronously growing MCF-7 cells were arrested by transfer to charcoal stripped medium. Quiescent cells were transfected with antisense p27 oligonucleotides (Asp27) or with missense oligonucleotides (MSp27) or with lipid only as controls. Within 7 hours of the onset of transfection, p27 levels were significantly reduced in Asp27 treated cells, while p21 levels were unchanged (Fig 5a). Thus the effects of the Asp27 were specific to p27 and did not influence directly the levels of p21. Despite the continued absence of estradiol, the p27 antisense treated group entered into S phase, with an S phase fraction rising from 5% at the onset of transfection, to 21% 21 hours after completion of transfection. By 28 hours post-transfection the ASp27 treated cells showed 29% cells in S phase. Control cells treated with lipid alone showed 5% and 6% S phase, while MSp27 treated populations had 9 and 11% S phase at 21 and 28 hours after completion of transfection.

G1 progression was also made evident in the ASp27 treated cells by phosphorylation of both p130 and pRb. In MSp27 and lipid only transfected controls these pocket proteins remained predominantly hypophosphorylated. The reduction of p27 protein in Asp27 transfected cells was sufficient to mediate loss of p27 from cyclin E-cdk2 complexes and activation of cyclin E-associated kinase. Both total cellular cdk2 (Fig 5b) and cyclin E-associated cdk2 (Fig 5c) in Asp27 treated cells showed a shift to the faster mobility form reflecting the thr160 phosphorylation that accompanies activation of this kinase. The progression of Asp27 cells into S phase was also accompanied by loss of p21 and loss of its association with cyclin E. It is worth noting that the loss of p21 also followed the loss of p27 as estradiol depleted MCF-7 were induced to enter cell cycle by re-addition of this steroid (Fig 1b).

Thus, the Asp27-mediated loss of p27 was sufficient to mimic the effect of estradiol on G1-to-S phase progression in MCF-7 cells. These data suggest that loss of p27 is critical for estradiol dependant stimulation of breast cancer proliferation and an increase in p27 is essential for cell cycle arrest following an inhibition of estradiol signaling. Ongoing studies will address further the mechanisms whereby estradiol influences p27 action and how the regulation of p27 may be are altered in steroid resistant breast cancer cells.

B) Specific comments on progress with the SOW (revised May 1998).

In section A) above, I have provided and discussed the significance of work that will soon be submitted for publication. In the present section, I will review the SOW to address aspects of the different tasks that have been completed and how the tasks proposed should be changed.

SOW TASK 2.

We tested the efficacy of transfection of the ASp27 oligos directly using western blotting for p27 as an end point (task 2 a completed). The ASp27 caused a significantly greater drop in p27 protein when it was introduced into quiescent cells (task2b completed). Very little loss of p27 was noted

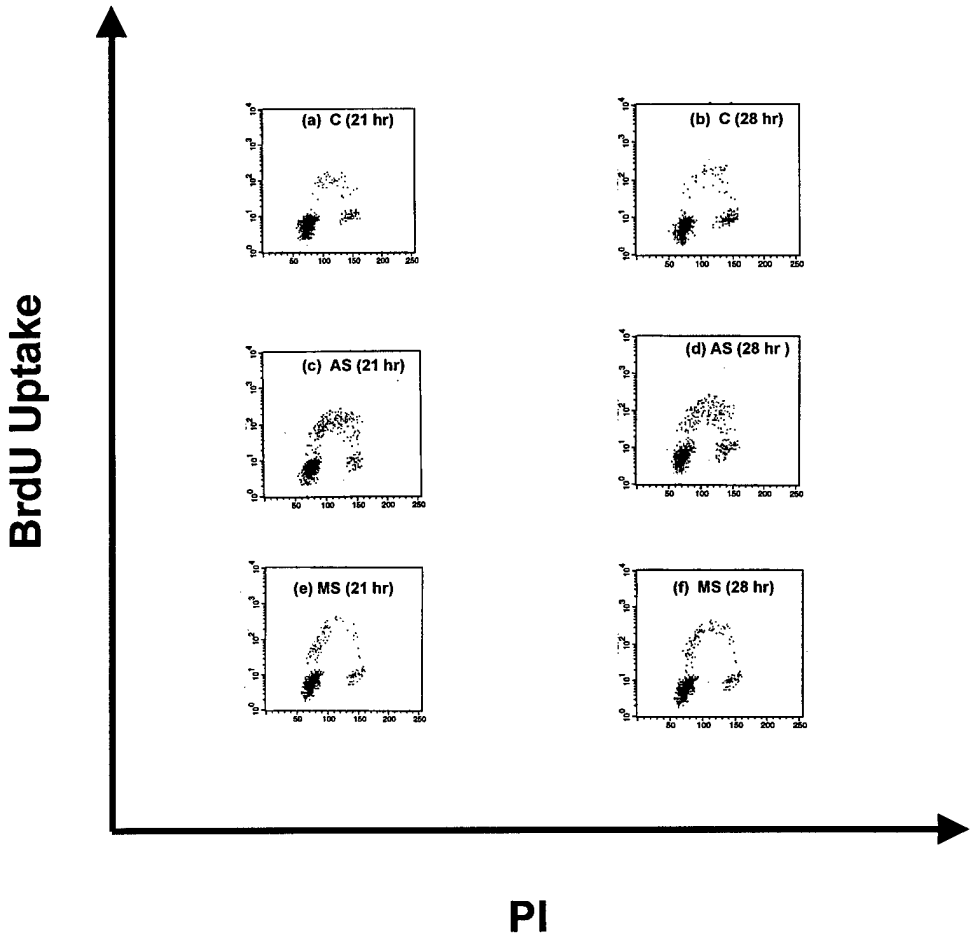


FIG. 4 Antisense p27 oligonucleotides stimulate exit from G0 in estradiol-depleted MCF-7 cells. Cells were estradiol depleted for 40 hours and treated for 6 hours with either antisense p27 oligonucleotides (ASp27), missense p27 oligonucleotides (MSp27) or lipid transfection vehicle alone (C) as controls. Cells were maintained in estradiol depleted medium. At 21 and 28 hrs after completion of transfection, cells were pulse labeled with BrdU and then stained with antiBrdU-conjugated FITC and counterstained with PI for flow cytometric analysis. DNA content is on the X axis and BrdU uptake on the Y axis of the graphs. ASp27 treated cells showed 21% and 29% S phase cells at 21 hrs and 28 hrs post-transfection, respectively, while control cells (C and MSp27) remained quiescent.

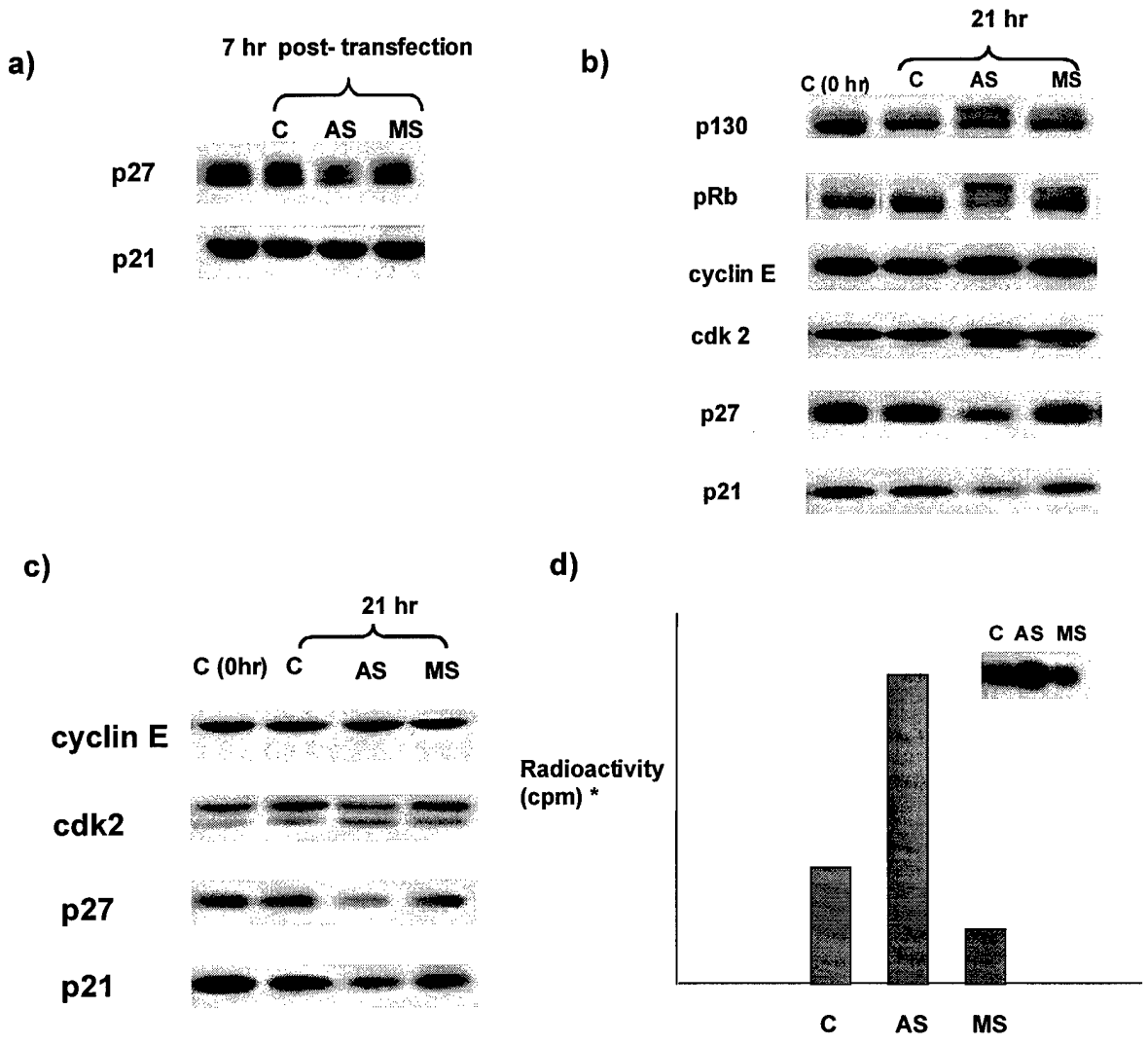


FIG. 5 Effects of ASp27 on cyclin E-cdk2 complexes and cyclin-E-associated kinase activity. *(a)* Western blot shows reduced p27 but no loss of p21 at 7 hours after the onset of transfection. *(b)* Cell cycle regulators were assayed by Westen blotting and *(c)* cyclin E complexes were assayed by immunoprecipitation blotting in C, ASp27 and MSp27 treated cells, 21 hrs after completion of transfection. Cyclin E complexes showed loss of associated p27 and p21 as ASp27 treated cells entered S phase. *(d)* Cyclin E-associated kinase was activated within 21 hrs of completion of ASp27 transfection.

when asynchronously growing cells were transfected. While it has been possible to show that p27 is essential for the maintenance of G0 induced by estradiol depletion, it is not possible to transfect actively proliferating MCF-7 to demonstrate whether Asp27 makes them resistant to arrest by estradiol depletion or by estrogen receptor blockade. Although we believe that p27 is probably required for the maintenance of G0 arrest induced by Tamoxifen or by ICI^{182780}, this will be formally tested within the next year. Thus, we anticipate the completion of Task 2 parts c and d within the next year. Our original plan was to carry out Task 2 in years 2 and 3. Instead it will be completed by the end of year 2.

SOW TASK 1.

This Task addressed work proposed in Specific Aim 2. We proposed to test the effects of proteasome inhibition on p27 levels. We have assayed the rate of synthesis and phosphorylation by metabolic labeling (both with ^{35}S methionine and orthophosphate) of p27 across the cell cycle in MCF-7. The rate of p27 synthesis detected on a 1 hour pulse labeling decreases as cells exit G0. Both p27 levels on Western blotting and the extent of orthophosphate labeling of p27 (see Figure 6) are increased by inhibition of the proteasome with the drug, LlnL. These data suggest that critical phosphorylation events precede degradation of p27 by the ubiquitin-dependent proteasome. Thus we have completed the work of Task 1a. In Task 1b and c, we had planned to use a temperature sensitive (ts) mutant of the ubiquitin activating enzyme E1 to determine how loss of this activity (loss of ubiquitin-mediated proteolysis) affects p27 levels, p27 phosphorylation and entrance into the cell cycle. However, while this was not well established in June 1997, it is now very clear from our own data and from recent work of Pagano's group and others (14-16), that p27 is indeed degraded by the proteasome. Thus inhibition of E1 activity using a ts E1 allele will undoubtedly increase p27 levels. Some of the work of Task 1b and c has been superceded by work published (14-16).

Some elements of Task1c remain to be pursued in a modified format (see revision proposed below). In pursuit of Specific AIM 1, our immunofluorescence studies have revealed that the localization of p27 changes across the cell cycle. Abundant nuclear p27 (stained green, Fig 7) is seen in estradiol depleted G0 cells. As cells approach the G1/S phase transition (T=12 hrs), 27 is transiently detected in the cytoplasm before its levels plummet as cells show strong BrdU labeling in S phase (BrdU stained red, see Fig 7). This suggests that export of p27 from nucleus to cytoplasm precedes degradation. Indeed recent work by others also suggests a connection between nuclear transport and p27 degradation (17). When we inhibit p27 degradation using LlnL, we can stabilize the cytoplasmic form of p27 (data not shown). Of particular interest is the observation that an ER positive steroid independent variant of MCF-7 cell line has abundant cytoplasmic p27 (data not shown). Thus, progression of breast cancer to estrogen independence may be linked to miss-localization and loss of function of p27 in these cells. Nuclear export of p27 may be critically regulated by phosphorylation events that mediate p27's association with components of the nuclear export machinery and lead ultimately to its degradation. Changes in p27 localization may reflect altered phosphorylation and loss of cyclin/cdk inhibitory function.

We propose to alter SOW Task 1 (see next page below). This proposed revision of Task 1 is a logical extension of our progress made in this grant to date. Increasingly, the regulation of function of components of the cell cycle machinery is being tied to changes in intracellular localization (18). It is important that the reviewers of this Progress Report keep these unpublished observations in strict confidence in order not to compromise my lab. It is also important that the logical pursuit of clearly important experimental leads not be foreclosed by an excessively rigid adherence to TASKS proposed in a 2 year old SOW that has now become somewhat obsolete. Insights into the regulation of p27 localization and degradation are germane to the loss of p27 protein seen in primary breast

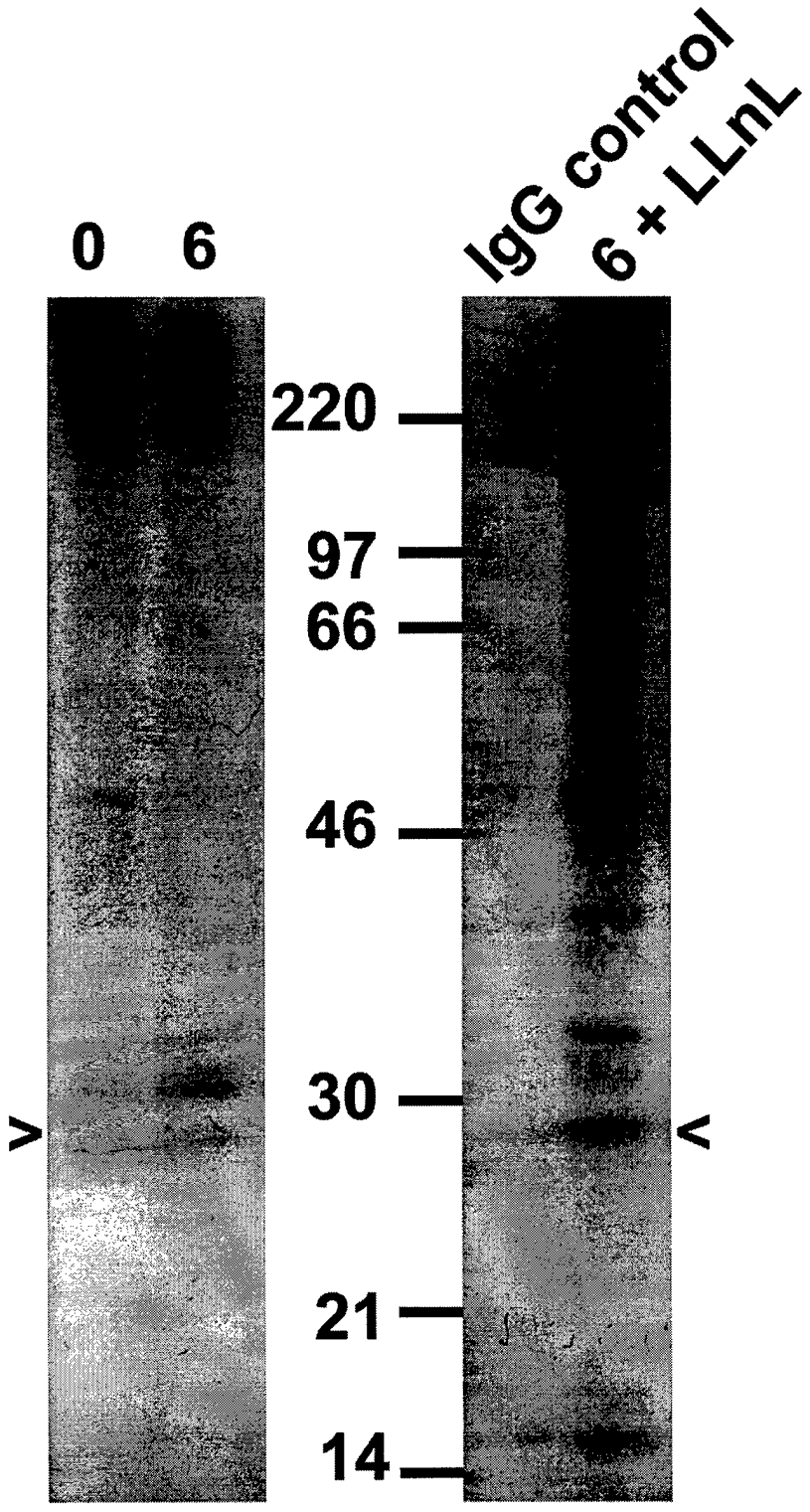


FIG. 6 Increased phosphorylation of p27 detected following treatment with the proteasome inhibitor, LInL. p27 was immunoprecipitated from orthophosphate labeled cells in G0 (t=0), or at 6 hours following release from G0 with or without pre-treatment with LInL. p27 is indicated by >.

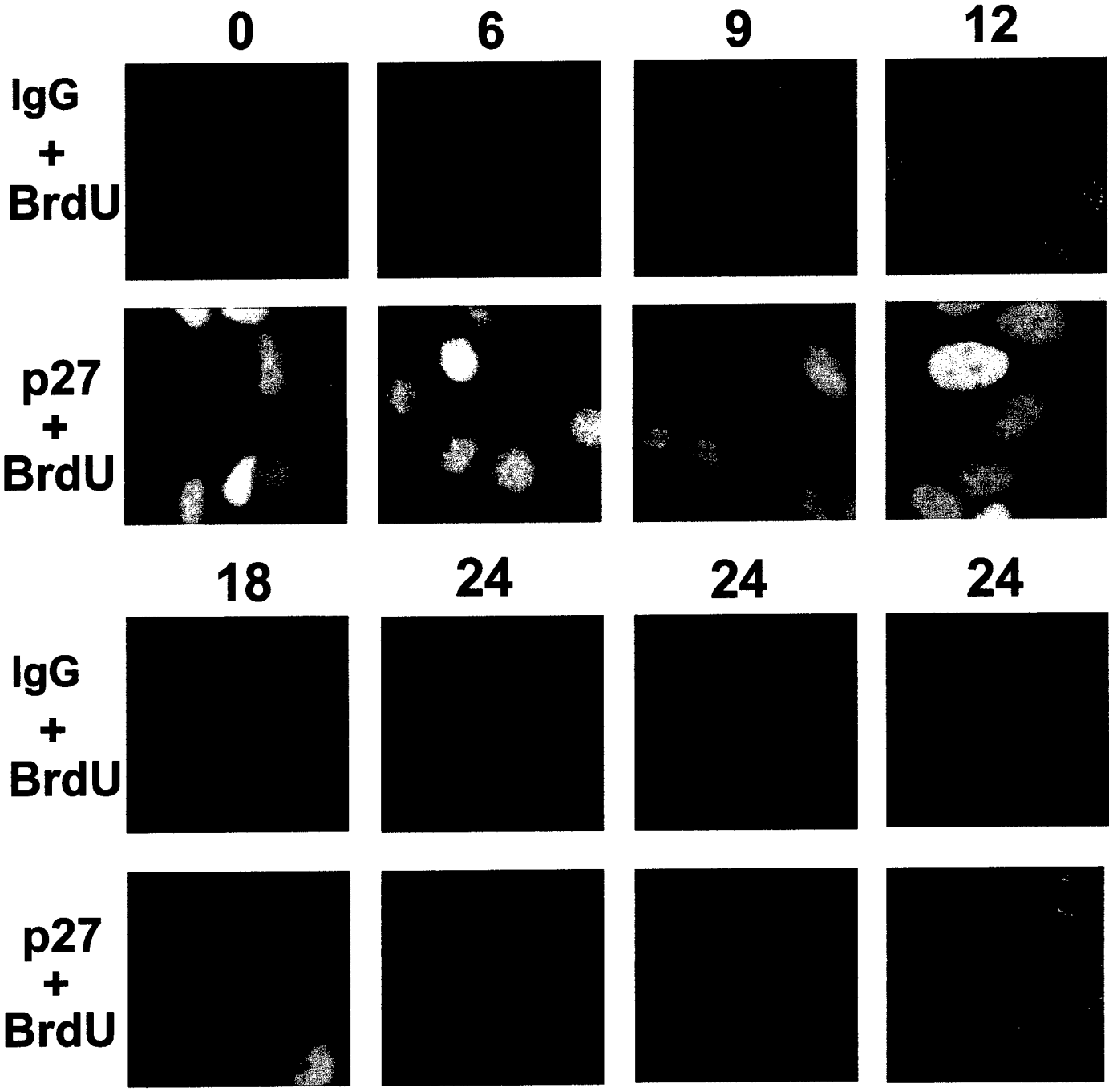


FIG. 7 Immunofluescence analysis of p27 and BrdU uptake during cell cycle progression. MCF-7 cells were plated on glass slides and synchronized by 48 hrs of steroid depletion. Cells undergo synchronous re-entry into the cell cycle on re-addition of estradiol. At intervals after addition of estradiol, cells were pulse labeled with bromodeoxyuridine (BrdU) then reacted with monoclonal anti-p27 antibody (Transduction Laboratories), then incubated with FITC-conjugated goat anti-mouse IgG. Cells were counter-stained with anti-BrdU antibody followed by Texas Red-conjugated to horse anti-mouse IgG. Cells were then photographed using a Ziess Axiophot fluorescence microscope. Nuclear p27 staining is maximal in G0 cells. The intensity of nuclear staining decreases with G1 progression. Transient cytoplasmic p27 staining can be appreciated as cells approach the G1-to-S phase transition at t=12hrs. p27 is lost during S phase (t=18-24 hrs) and re-accumulates in the nucleus in G2 (24 hrs). Lower panels (p27 + BrdU) show p27 and BrdU staining. Top panels (IgG + BrdU) represents controls for p27 staining where primary anti-p27 antibody was

cancer. We saw a strong association between reduced p27 protein levels and progression to steroid independence in primary breast cancers (8). We have shown, in the work of the last year, that p27 is essential for the maintenance of growth arrest of breast cancer cells following interruption of estradiol signaling. A better understanding of how this key effector, p27, is regulated and how these processes are altered in breast cancer progression may shed new insights on the problem of progression to Tamoxifen resistance in breast cancer.

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Appendix 1

Proposed revision of SOW for grant award numbers DAMD17-98-1-8158 and DAMD17-98-1-8159 (revised July1999)

PI: J. M. Slingerland

TASK 1 (revised)

1) Assay effects of LlnL on p27 levels and its intracellular localization (year 1, done)

2) Carry out nuclear cytoplasmic fractionation and test whether p27 phosphorylation differs in the cytoplasmic and nuclear fractions using 2 dimensional immunoelectrophoresis techniques that have been established in the Slingerland lab (year 2)

3) Test whether in vivo associations between p27 and components of the nuclear export machinery (export carrier proteins, CRM1, importin beta, transport proteins Ran and RanGAP) are detectable and whether they vary across the cell cycle. (8 months required in year 2)

4) Test the effects of inhibition of the proteasome on the levels of p27 binding to nuclear export factor CRM1 (2-3 months in year 2).

5) Show whether the ability of p27 immunoprecipitated from MCF-7 to bind recombinant CRM1 varies as a function of cell cycle progression and is associated with differences in p27 phosphorylation (year 3).

6) Investigate how MCF-7 variants that have become steroid independent differ in their regulation of p27 localization and proteolysis (year 2 and 3).

TASK 2 remains unchanged and will be completed by the end of year 2 of the grant period

Appendix 2

J. Slingerland

Revised Statement of Work May 1998

In the context of a separate grant, Burrough's Wellcome Fund (see appended abstract) we will compare effects of antiestrogen and estrogen on the cell cycle and compare effects of these agents on p27 protein expression, stability, p27 phosphorylation, localization and binding of novel p27-associated proteins in steroid sensitive and insensitive breast cancer lines which express the ER. **The work above will complement the work outlined below to be carried out in the context of grants BC972846 and BC972843 funded by the DOD as follows:**

Task 1 (formerly Task 2): To test whether increased ubiquitin proteasome activity lowers p27 levels in estradiol stimulated MCF-7 cells

a) We will use the chemical proteosome inhibitor, peptide-aldehyde N-acetyl-leucinyl-leucinyl-norleucinal-H (LLnL) in estradiol stimulated MCF-7 cells to visualize the unstable forms of ubiquitin-bound p27 following estradiol stimulation of MCF-7; **months 1-8**

b) Transfection of a vector bearing temperature sensitive (ts) mutant of the ubiquitin activating enzyme E1 into MCF-7; **months 2-6**

c) to determine how loss of this ubiquitin activating enzyme (E1) activity affects p27 levels, p27 phosphorylation and entrance into the cell cycle; **months 8-36**

Task 2 (formerly Task 3, revised to include non-overlapping investigations) We will determine whether p27 is critical to growth inhibition by antiestrogens using antisense p27 oligonucleotides in MCF-7 in an effort to make MCF-7 resistant to arrest by antiestrogens.

a) To test the efficacy of delivery/transfection of the antisense oligos, we will optimize delivery of a beta galactosidase marker **months 12-18**

b) Test efficacy of p27 antisense oligos to inhibit p27 expression in MCF-7 and possibly ZR-75-1; **months 18-36**

c) Test whether antisense p27 oligos cause loss of the ability to upregulate p27 protein following treatment with Tamoxifen; **months 18-36**

d) Test whether loss of p27 up-regulation by Tamoxifen in cells treated with antisense p27 oligos renders cells resistant to growth inhibition by Tamoxifen; **months 18-36**

The technician will carry out Task 2 and assist with Task 1 while Dr. S. Cariou (50% effort on this grant) will do the work of Task 1 and assist with Task 2. I will direct their work and involve myself in bench work as time permits.

Appendix 3

Slingerland, J.M.

Statement of Work

Task 1: Compare effects of antiestrogen and estrogen on steroid sensitive and insensitive breast cancer lines which express the ER

a) We will test whether phosphorylation of p27 precedes the reduction in p27 levels following estradiol stimulation of steroid sensitive breast cancer lines; **months 1-12**

Western analysis of p27 and FACs analysis across cell cycle in estradiol stimulated MCF-7 and ZR-75-1 will be completed in 1-2 months.

Metabolic labeling and immunoprecipitation of p27 using ^{35}S methionine and ^{32}P-orthophosphate to show estradiol induced changes in both phosphorylation and half-life of p27; completed months 2-12

b) We will test whether estradiol effects on p27 are blocked by antiestrogens; **months 6 to 24**

Asynchronously growing cell will be treated with TAM and ICI182780, cell cycle effects documented by FACs, p27 protein levels, phosphorylation and half-life will be assayed. immunofluorescence studies of p27 localization will be carried out using protocols already developed.

c) How estradiol sensitive and resistant lines differ in p27 protein expression, stability, p27 phosphorylation, localization and binding of novel p27-associated proteins; **months 12-36.**

Steroid resistant LY2 and ZR-75-1-5B lines will grown, methods of achieving synchronization in the cell cycle will be developed

Cell cycle regulators will be compared in sensitive and resistant cell line (cyclin, cdk, and cdk inhibitor expression, complex formation assayed by IP/Western and kinase assays completed)

p27 protein levels, phosphorylation and half-life and immunofluorescence studies of p27 localization will be will be compared in sensitive and resistant lines using protocols already developed.

Task 2: To test whether increased ubiquitin proteasome activity lowers p27 levels in estradiol stimulated MCF-7 cells

a) We will use the chemical proteosome inhibitor, peptide-aldehyde N-acetyl-leucinyl-leucinyl-norleucinal-H (LLnL) in estradiol stimulated MCF-7 cells to visualize the unstable forms of ubiquitin-bound p27 following estradiol stimulation of MCF-7; **months 12-18**

b) Transfection of a vector bearing temperature sensitive (ts) mutant of the ubiquitin activating enzyme E1 into MCF-7; **months 12-14**

c) to determine how loss of this ubiquitin activating enzyme (E1) activity affects p27 levels, p27 phosphorylation and entrance into the cell cycle; **months 18-36**

Task 3. We will determine whether p27 is critical to growth inhibition by antiestrogens by introducing inducible antisense p27 into MCF-7 or by using antisense p27 oligonucleotides in an effort to make MCF-7 resistant to arrest by antiestrogens.

a) Construction and testing of p27 antisense vectors; **months 1-6**
b) Introduce tTA bearing plasmid into MCF-7 clones and test for tight inducibility; **months 1-12**
c) Introduce anti-sense p27 into tightly inducible tTA-bearing MCF-7 clones and test effects of tet withdrawal on p27 expression by western and effects of loss of p27 on cell viability and on cycling; **months 12-24**
d) Test whether tet withdrawal causes cells to become resistant to antiestrogens; **months 24-30**
e) Test efficacy of p27 antisense oligos to inhibit p27 expression in MCF-7 and possibly ZR-75-1; **months 18-36**

The technician will carry out Task 3 while my post-doctoral fellow, Dr. S. Cariou, completes the work of Tasks 1 and 2. I will direct their work and involve myself in bench work as time permits.

List of Key Research Accomplishments

1. Completed analysis of G1 cyclin/cdk complexes and their kinase activities in breast cancer cells synchronized to quiescence by steroid depletion and released into the cell cycle by re-addition of estradiol.

2. Demonstrated p27 localization by immunohistochemistry and p27 localization and timing of DNA synthesis by immunofluorescence using dual labeling of p27 and BrdU uptake.

3. Characterized kinetics of cell cycle arrests and profiles of cyclin/cdk complexes and kinase inhibition in MCF-7 cells induced to G1 arrest by anti-estrogens ICI^{182780}, Tamoxifen, and by estradiol depletion.

4. Achieved good efficiency of tranfection of MCF-7 cells with antisense p27 oligonucleotides and appropriate controls.

5. Used antisense p27 oligonucleotides to show that p27 is essential for maintenance of G1 arrest following interruption of estradiol signaling in steroid sensitive breast cancer cells.

6. Devised effective protocols for metabolic labeling of p27 using ^{35}S-methionine and orthophosphate

7. Showed that levels of p27 and p27 phosphorylation both increase following inhibition of proteolysis using LInL (a proteasome inhibitor).

8. Demonstrated that p27 is strongly nuclear in G_0, exits the nucleus and can be detected transiently in the cytoplasm at the G1/S transition. p27 is then degraded in S phase and re-accumulates in nucleus and in G2.

9. Achieved effective nuclear-cytoplasmic fractionation in MCF-7 cells.

10. Demonstrated that inhibition of p27 proteolysis allows detection of increased p27 in the cytoplasmic fraction of MCF7 cells. (Normally, only nuclear p27 is detected in MCF-7, likely because p27 gets degraded as soon as it enters the cytoplasm)

List of Reportable Outcomes

Manuscripts

1. Articles

   p27 is essential for cell cycle arrest following interruption of estradiol signaling in MCF-7 breast cancer cells. S. Carious, J. Donovan, N. Bhattacharya and J. Slingerland, in preparation for PNAS, 1999.

2. Review article

   Deregulation of the Cell Cycle in Cancer. S. Sandhu and J. Slingerland, in press, Cancer Detection and Prevention, 1999.

Abstracts and Presentations

1. Plenary Talks at International Meetings

   "Regulation of the Cell Cycle Inhibitor p27 by Estrogens and Anti-estrogens", J. Donovan, S. Cariou, N. Bhattacharya, J. Slingerland. Presented at the Breast Cancer Research: Reasons for Hope, National Scientific Conference, Toronto, June 17-19, 1999.

   "Estrogens and Prevention" (Early Events in Cellular Proliferation & Pleitropic Transmembrane Signaling). Presented at the Satellite Conference on Breast Cancer meeting, Ottawa, July 26-28, 1999.

2. Poster Presentation

   "Regulation of the Cell Cycle Inhibitor p27 by Estrogens and Anti-estrogens", J. Donovan, S. Cariou, N. Bhattacharya, J. Slingerland. Presented at the Salk Institute Cell Cycle Meeting, San Diego, CA, June 18-22, 1999.

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Pre-doctoral Fellowship Award Submitted by J. Donovan to US Army MRMC Breast Cancer Program, June 2, 1999

Deregulation Of The Cell Cycle In Cancer

Charanjit Sandhu and Joyce Slingerland M.D., Ph.D.*

Division of Cancer Biology Research, Sunnybrook and Women's College Health Science Centre, Toronto, Ontario, Canada[1]

* Corresponding Author. Mailing address: Division of Cancer Biology Research, Sunnybrook and Women's College Health Science Centre, S-218, 2075 Bayview Avenue, Toronto, ON M4N 3M5, Canada. Phone: (416) 480-6100 Ext. 3494. Fax: (416) 480-5703.
E-mail: joyce.slingerland@utoronto.ca.

Running Title: Deregulation Of The Cell Cycle In Cancer
Key Terms: Cancer, Cell Cycle, Cdk Inhibitors

Introduction

In the past decade, significant advances have been made in our understanding of the mitogens that stimulate growth, the receptors to which they bind, the components of mitogenic signaling pathways and finally, the effectors of proliferative changes, the cell cycle regulators. The cell cycle regulators, the cyclins and cdks were, for the most part, initially identified in yeast[1]. Many of the human cyclin/cdk homologs were identified by virtue of their ability to rescue yeast mutant phenotypes[2-4]. This review will provide a brief overview of mammalian cell cycle regulation and how it becomes deregulated in cancers.

A brief overview is provided of the complexes that govern transitions from one phase of the cell cycle to the next: the cyclins, cyclin dependent kinases(cdks), cdk inhibitors and phosphorylation events that regulate cdk activity. In the final segment, we review the alterations in cell cycle regulators found in human cancers and summarize the data bearing on the prognostic significance of these changes.

Cell Cycle Overview

In eukaryotic cells, cell cycle progression is coordinated by a tightly regulated series of events. The cell cycle involves four different phases: G1, S, G2 and M phase. Following mitosis (M phase), the daughter cells enter the G1 phase, where cell growth primarily occurs. It is during the G1 phase that the cell is responsive to growth inhibitory or mitogenic signals. The G1 phase is followed by the S phase, in which DNA replication occurs. This is immediately followed by the G2 phase, during which the integrity of DNA replication is verified. The cells then reenter M phase and undergo mitosis and cytokinesis to produce two daughter cells. In tissue culture, cells can exit the cell cycle upon the removal of serum or growth factors and enter a quiescent or G0 state, which is characterized by a low rate of metabolic activity and sensitivity to mitogens.

Movement of the cell through the cell cycle requires the activity of a family of cyclin dependent kinases (cdks). The cdks consist of a family of related serine/threonine kinases, cdks 1 to 7 whose members require the association with a cyclin for catalytic activation[5, 6]. Different cyclins bind and activate different cdks. The transition from the G1 phase into S phase requires the phosphorylation of the retinoblastoma protein (pRb) which is mediated primarily by cyclin D1 associated cdk4 or cdk6, and also by cyclin E-cdk2[7, 8]. Phosphorylation of pRb releases associated E2F-DP1 heterodimers [9, 10]. Members of the E2F transcription factor family are then free to activate the expression of various genes that are required for entry and progression through S phase[11].

Progression through G1, and entrance and progression through S phase are dependent upon cdk2 activity[12-15]. In late G1 and early S phase, cyclin E-cdk2 and the more recently discovered cyclin E2/cdk2 phosphorylate various targets which are required for S phase entrance[16-18]. Cyclin D1 and E-type cyclins are essential for the movement of the cell through the G1/S transition. Microinjection of antibodies to cyclin D1[19], cyclin E1[20] or cyclin E2[17] can inhibit G1 to S phase progression. Overexpression of cyclin D1 or cyclin E shortens the G1/S phase interval[21-23]. Moreover, simultaneous coexpression of both cyclins further shortens the G1 phase, suggesting that cyclin D1 and E complexes may have distinct targets[24]. In late S phase, cdk2 becomes predominantly associated with cyclin A. The activity of cyclin A-cdk2 is essential for entrance to and progression through S phase[25, 26]. Movement through the final cell cycle checkpoint at the G2/M transition is mediated by the activity of cdk1 in association with partners of the cyclin B family[1, 6].

Cdk Activation By Cyclins

As the name cdk implies, the binding of a cyclin is an absolute requirement for the catalytic activation of the cdks. The cyclins consist of a family of proteins sharing a conserved

region of approximately 100 amino acids, referred to as the cyclin box[5]. The cyclin box serves as a docking site for the recruitment of cdk inhibitors or cdk substrates to cyclin/cdk complexes. Cyclin binding leads to conformational changes within the cdk that are required for its activation[27].

The precise timing of cyclin/cdk activation during the cell cycle ensures that the complexes are catalytically active only when required[28]. This is regulated in part by the specific subcellular localization and the timed expression of the various cyclins throughout the cell cycle. In general, peak nuclear expression of a specific cyclin occurs when peak activity of the partner kinase is required.

Cyclin levels are regulated by ubiquitin–mediated proteolysis[29]. The cyclins are rapidly exported from the nucleus and degraded when not required[30-33]. For example, the cyclin D1 protein accumulates in the nucleus during G1 and exits the nucleus and is degraded during late G1 and S phase[19, 33, 34]. Similarly, nuclear import and export of B type cyclins are actively regulated in a cell cycle dependent manner. Phosphorylation of specific sites in cyclin B1 are required for association of a nuclear export factor CRM1[31, 35].

Cyclins are also subject to important regulation at the level of transcription. Growth factors have been shown to stimulate cyclin D1 synthesis and their removal reduces its mRNA synthesis[36-38]. Cyclins E, A, and B are also transcriptionally regulated, with peak mRNA expression coinciding with peak kinase activities[5].

Cdk Regulation by Phosphorylation

Specific phosphorylation and dephosphorylation events are required for cdk activity[39]. A threonine 160 (thr-160) residue within the T loop of cdk2 (thr-161 in cdk1, and thr 172 in cdk4) must be phosphorylated for the kinase to be active[40, 41]. Phosphorylation of this residue induces conformational changes that reduce steric hindrance of substrate binding[42]. The cdk activating

kinase, or CAK, catalyzes phosphorylation of the thr-160 site. In mammalian cells, CAK is a complex of Cdk7 in association with cyclin H. A relatively low conservation of the cyclin box sequence in cyclin H precludes the binding of KIP inhibitors to this cyclin[43, 44]. Consequently, the KIPs do not directly regulate the activity of cdk7/cyclin H complexes (CAK). However, the KIPs may regulate substrate availability for CAK complexes, since the association of KIPs to cyclin/cdks impedes access of CAK to the catalytic cleft of the cdk moiety[45].

Phosphorylation of threonine-14 (thr-14) and/or tyrosine-15 (tyr-15) residues inactivates cdk1 and cdk2, and analogous sites exist on cdk4[40, 46-49]. The human homologs of the yeast wee1 and the xenopus myt1 phosphorylate cdc2 at thr-14 and tyr-15[50-53].

Members of the Cdc25 phosphatase family, Cdc25A, Cdc25B and Cdc25C, remove the inhibitory phosphates from the cdks[54-58]. These phosphatases are periodically expressed and catalytically active at discrete times within the cell cycle. Cdc25A levels are maximal at the G1/S transition and this phosphatase targets cyclin E/cdk2[59, 60]. Cdc25A may also act on cdk6[49]. Cdc25A expression is induced in certain cell types by c-myc and Cdc25A may be phosphorylated and activated by Raf1 or by cyclin E/cdk2[59, 61, 62]. Cdc25B and Cdc25C are predominantly active at the G2/M transition and act on mitotic cyclin B-associated cdk1[63].

Cdk Inhibitors Regulate Cdk Activity

The cdk inhibitors consist of two families: the **In**hibitors of cd**k4** (INK4) and the **k**inase **i**nhibitor **p**roteins (KIP)[64]. The KIP family consists of three broadly acting inhibitors $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. KIP family members bind to and inhibit the cyclin-cdk complexes[65-67]. In vitro experiments demonstrate that a single molecule of p21 or p27 is sufficient to inhibit the kinase activity of a cyclin/cdk complex[68]. The affinity with which these KIPs associate with target cyclin/cdks may be regulated by phosphorylation[69, 70]. Inhibition of cyclin D and cyclin E associated kinases by KIPs results in a G1 cell cycle arrest. p27 was first identified in cells

arrested by transforming growth factor-β (TGF-β) , by contact inhibition and by lovastatin[71-74]. p27 is essential for G0 arrest resulting from serum withdrawal[75] and upon estradiol withdrawal from estrogen dependent breast cancer cells (Donovan and Slingerland, Manuscript in preparation). An increase in the association of p27 with its target cyclin/cdks occurs in response to a number of anti-proliferative signals. Levels of both p27 and p21 are regulated by ubiquitin-mediated proteolysis[76-78](Carrano and Pagano, in press). p27 is also subject to translational controls[79, 80]. p21 is induced by many forms of cellular stress and by DNA damage and p21 upregulation serves to coordinate cell cycle arrest with mechanisms of DNA repair[81-83]. In addition to their role as cdk inhibitors, the KIPs may also facilitate assembly and localization of cyclin D1/cdk complexes within the nucleus[84, 85].

INK4 family of inhibitors

In contrast to the KIP inhibitors, the inhibitory activity of INK4 family members, $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$ are restricted to cdk4 and cdk6[64](reviewed by[86]). The *p16*^{INK4A} gene was discovered as a cdk4 associated protein[87] and as the MTS1 gene targeted by chromosomal deletions in many human cancers[88]. A tumor suppressor role for p16 is supported by *p16*$^{-/-}$ mice, which have a substantially increased incidence of cancers when compared to wild type mice[89]. *p15*^{INK4B} was cloned as a gene induced in response to TGF-β[90]. In epithelial cells, G1 arrest by TGF-β involves induction of the $p15^{INK4B}$ gene, stabilization of the $p15^{INK4B}$ protein and accumulation of $p15^{INK4B}$ in cdk4 and cdk6 complexes[91, 92]. p16 levels are increased in senescent fibroblasts and p16 plays a role in the arrest of cellular proliferation at senescence[89, 93, 94].

Cell Cycle Deregulation in Cancers

The aberrant expression of cell cycle regulators has been identified frequently in human cancers. Cell cycle regulators that promote cellular proliferation, such as G1 cyclins and Cdc25

phosphatases, show elevated expression while the negative regulators of cell cycle progression are frequently functionally inactivated by deletion, mutation or show a reduction in protein levels, often without any directly associated genetic changes (Table 1). Changes in the levels of cyclins D1 and E and loss of the cdk inhibitor p27 protein in human cancers may have significant prognostic implications for patient outcome. The following provides a brief summary of the alterations in cell cycle regulators that have been identified in human malignancies.

Upregulation of Positive Effectors

In cancers, increased cyclin expression may result from translocation, gene amplification, or mutation. The levels of cyclins D1 and E are frequently increased in cancers[95-101]. Constitutive G1 cyclin expression may serve not only accelerate the G1 to S phase transition but may also make cancer cells less responsive to growth inhibitory stimuli. In the context of genomic instability in evolving pre-malignant lesions, the increased proliferation may promote clonal outgrowth and tumor progression. Both cyclins D and E can cooperate with *ras* in transformation assays in tissue culture. In transgenic mice, overexpression of cyclin D1 under the control of the mouse mammary tumor virus promoter, resulted in mammary hyperplasia and mammary adenocarcinomas[102].

Cyclin D1 mRNA and/or protein levels are frequently elevated in cancers and hematologic malignancies[99, 103, 104]. Chromosomal translocations leading to an increase in cyclin D1 have been reported in parathyroid tumors and follicular lymphomas[104-106].

Both cyclin E and D have been found to be amplified in different carcinomas[95, 103, 107-109]. In addition to translocation and amplification, increased cyclin D1 and cyclin E expression have been observed without evidence for genetic changes[98, 110, 111]. Despite the oncogenic potential of cyclin D1 in tissue culture and in mice, overexpression of cyclin D1 in human breast, which is correlated with expression of the estrogen receptor[112] has yet to be consistently correlated with

an unfavorable prognosis[113-117]. Overexpression of cyclin E is associated with a poor prognosis in breast cancer[110, 118]. Mutations that affect cyclin stability have been reported. An integration of the hepatitis B virus within the cyclin A gene region encoding the cyclin's destruction box produced a highly stable protein in a hepatocellular carcinoma[119, 120]. Of note, overexpression of cyclin B has not been observed in human cancers. In cell culture, constitutive expression of cyclin B leads to arrest at mitosis[121, 122]. The requirement for destruction of cyclin B for exit from mitosis likely accounts for the lack of over-expression of this cyclin in cancers.

Translocation and amplification events that increase the expression of cdk4 in cancers have also been reported in sarcomas and carcinomas of the nervous system[123]. In breast cancers, 15% of tumors examined have shown to contain amplification of the cdk4 gene[124]. In addition, cdk4 mutations that produce a dominant active kinase have been identified in melanomas[125]. To date, no mutations in other cdk genes have been identified in human cancers. Other promoters of cell cycle progression, such as the Cdc25 family of phosphatases, may be overexpressed in cancers[126]. Cell culture experiments have demonstrated the oncogenic potential of Cdc25 phosphatases. Ectopic overexpression of *Cdc25A* or *Cdc25B* with oncogenic *ras* can transform cultured mouse embryo fibroblasts[127]. In human breast cancers, overexpression of Cdc25A has been detected in a third of tumor samples[127]. Loda et al have observed an association between high levels of Cdc25A with a poor breast cancer patient prognosis (M. Loda, personal communication).

Loss of Cdk Inhibitors

Mutations or deletion of the members of the INK4 gene family are not uncommon in many human malignancies. p16 in particular, plays a tumor suppressor role. In human cancers, the *p16* gene is frequently deleted or mutated[88, 128, 129]. Although somewhat less common that deletions, tumor-associated *p16* mutations have been identified that encode defective p16

proteins. Such mutant p16 proteins are unable to bind and inhibit their cdk targets[130-133]. These *p16* point mutations are frequently associated with familial melanomas. Silencing of the *p16* gene can also occur through hypermethylation of the *p16* promoter in human cancers[134]. Deletions of *p15* often accompany the loss of *p16* due to their close proximity on chromosome 9p. *p15* and *p16* deletions are fairly common in acute lymphoblastic leukemia. Several groups have examined the prognostic value of *p16* deletions in pediatric acute lymphoblastic leukemia(ALL), however, these studies fail to arrive at a consistent conclusion regarding the prognostic value of p16[135-137].

The prognostic value of p21 protein levels is somewhat controversial in cancers. Of five studies that examined p21 protein in primary breast cancer, two reported that high tumor p21 expression was associated with a poor prognosis[138, 139], two other studies have reported the opposite result [140, 141], and a fifth study showed no prognostic value associated with p21 in breast cancers[142]. The inconsistent conclusions may be attributable to the lack of uniformity in methods used to score p21 expression or to differences in distributions of tumor stages in the populations studies, see Tshilias et al. for review[143]. In other cancers, the prognostic role of p21 is also unclear. In gastric carcinoma, two studies demonstrated that tumors with low p21 expression were associated with a poor prognosis[144, 145], while in squamous cell carcinomas of the head and neck, high levels of p21 expression were associated with a shorter predicted disease free survival[146].

Of the aberrations in cdk inhibitors studied in human cancers to date, loss of p27 may have the greatest potential as a clinically relevant prognostic factor. The *p27* gene is rarely mutated or deleted in cancers[147-150]. However, while normal epithelial tissues show high nuclear levels of p27 protein, primary cancers of the breast, colon, lung, prostate stomach and esophagus

frequently show loss of p27 protein, associated with reduced time to disease relapse and or reduced survival (reviewed in[151]).

Three independent studies of differing cohort compositions have all identified p27 to be an independent prognostic factor in primary breast cancers[118, 152, 153]. Tan et al examined the prognostic value of p27 in a cohort of 202 patients with early stage breast cancer whose tumor size did not exceed 1cm. Low levels of p27, defined as less than 50% of the tumor nuclei staining positive for p27, were observed in approximately half of the tumors studied. Multivariate analysis identified loss of p27 as an independent prognostic factor for increased mortality (relative risk of death is 3.4, CI 1.12-10.3, p=0.03)[153]. A cohort representative of the whole breast cancer patient population was evaluated by Catzavelos et al.[152]. In this cohort of 168 patients the mean patient age was 62 years and 48% of the cases were node negative. Using the same scoring criterion as Tan et al., this study also found low p27 staining in 56% of the tumors and loss of p27 was a strong independent predictor of reduced disease-free survival. A low level of p27 was associated with a 2.1 fold increase in the risk of disease recurrence (CI 1.2-4.0, p=0.02).

Porter et al. assayed p27 by immunohistochemistry in the primary breast cancers of 246 premenopausal women under 45 years of age[118]. Unlike the two studies above, in this study, p27 levels were scored as low, intermediate or high. Both p27 and cyclin E levels were assayed. Multivariate analysis demonstrated that both low levels of p27 (relative risk of death 2.7, p=0.01) and high levels of cyclin E (relative risk of death 2.4, p=0.03) were independent predictors of overall survival. In addition, patients whose breast cancers showed both low p27 and elevated cyclin E proteins had the highest mortality (RR 8.6, 95%CI 3.6-20.4, p<0.001)[118].

In colon, prostate and gastric carcinomas, reduced p27 levels in the tumor specimen have also been correlated with poor patient prognosis[154-156]. In prostate cancers, three studies have

concluded that reduced levels of p27 are associated with a lack of organ confinement at radical prostatectomy and predicted reduced time to treatment failure[154, 157-159]. Similarly, in colon cancer loss of p27 in primary tumor is of independent prognostic value[155]. In addition to reduction in the level of p27 protein in cancers, a change in the subcellular localization of p27 may also be of prognostic significance. The localization of p27 within the cytoplasm as opposed to the nucleus was associated with a poorer survival in a study of Barrett's Associated Adenocarcinoma (BAA) of the esophagus[160]. While most of the cancer specimens examined to date show nuclear staining or loss thereof, increased attention must be given to p27 localization in cancers. Future studies examining the prognostic value of changes in cell cycle regulators may need to evaluate the significance of both expression levels and localization of these molecules.

While loss of p27 might predict an increased proliferative fraction in the tumor, not all studies show such an association[118, 152, 153, 161, 162]. Reduced p27 protein in human cancers is strongly associated with increased tumor grade. Tumors with reduced p27 show are often poorly differentiated in comparison to their counterparts expressing higher levels of p27[151, 154, 157, 163]. A link between p27 and cellular differentiation is also suggested by studies if p27 null mice. $p27^{-/-}$ mice develop parathyroid tumors, and manifest multiorgan hyperplasia and aberrations in cellular differentiation[164-166]. Thus, p27 may not only function to regulate cellular growth but also play a role in cellular differentiation[167-169].

The reduced level of p27 in human cancers may reflect increased degradation of the protein via the unbiquitin pathway. Extracts derived from colon, breast, lung and esophageal cancers with low levels of p27 protein have demonstrated an elevated proteolytic capacity towards recombinant p27 in vitro[153, 155, 160, 170]. Alterations in oncogenic signaling pathways may

lead to increased activation of the proteolytic machinery which lead to reduced levels of p27 in tumors.

Conclusions

The frequency with which cell cycle deregulation is observed in human cancers is not surprising given the critical role that cell cycle regulators play in promoting or inhibiting cellular proliferation. Genetic changes or changes in the expression levels of cyclins and cdk inhibitors may ultimately be found to be clinically useful as prognostic markers in human cancers. Studies evaluating the prognostic value of p27 have yielded the most consistent results to date. Immunohistochemical evaluation of p27 may ultimately prove clinically useful in the assessment of treatment options for patents with many forms of adenocarcinoma, including breast, colon, lung, prostate and gastric carcinoma. Immunohistochemical staining for p27 is relatively inexpensive and can be efficiently carried out in large numbers of tumors samples using automated stainers. Contradictory results from studies of p21 in human cancers reflect small sample sizes and inconsistent methodology in the scoring of p21 levels. Movement of p21 and p27 immunostaining into the clinical arena will require the adoption of a common criteria to define tumors as expressing either elevated or reduced levels of the protein of interest. Clarification of the prognostic role of *p16* deletions in hematologic malignancies will require larger cohorts and prospective studies. Although the value of a prognostic factor in cancers for which there are no curative therapies is limited, an increased understanding of the importance of cell cycle regulators in cancers may lead to the development of pharmaceutical antagonists or homologs that would assist in the treatment of cancers.

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Table 1. Cell cycle regulators are deregulated in human cancers

Cell Cycle Regulator	Alterations (Genetic/Protein)	References
[1]Cyclin D1	Amplification, Translocation, Increased Protein	96,97,98,99,100,101,104,106-110, 114-118
Cyclin E	Amplification, Increased Protein	96,97,104,110,111,119
Cyclin A	Viral Integration, Amplification	121, 102
Cdk4	Amplification, Translocation, Point Mutations	124,125,126
[2]$p27^{KIP1}$/$p21^{CIP1}$	Deletion, Rarely Mutations, Decreased Protein	139-143,145,146,148,149,153-161,163
[3]$p15^{INKB}$/$p16^{INK4A}$	Deletion, Point Mutations, Hypermethylation, Decreased Protein	89,129,130-133,135-138

[1]see review by Hall et al.(101)
[2]see review by Cariou et al.(152)h
[3]see review by Tsihlias et al.(155)

Regulation of the Cell Cycle Inhibitor p27 by Estrogens and Antiestrogens

Jeffrey Donovan, Sandrine Cariou, Nandita Bhattacharya and Joyce Slingerland

Division of Cancer Biology Research, Sunnybrook and Women's College Health Science Centre, 2075 Bayview Avenue, Toronto, Ontario, Canada.

Estradiol is a regulator of cell proliferation in mammary cells. 70% of breast cancers express the estrogen receptor, (ER) and two thirds of these are responsive to Tamoxifen. Both estrogens and antiestrogens influence the cell cycle regulators during the G1 phase. In breast tumors, loss of p27 is associated with a more malignant phenotype; we postulate that altered p27 degradation in breast cancer may contribute to resistance to antiestrogen therapy.

Cell cycle effects of estradiol were assayed in the ER+ breast cancer cell line, MCF-7. MCF-7 is strongly growth inhibited by the removal of estradiol or by the addition of Tamoxifen or the non-steroidal ER-blocker, ICI182780. Upon re-addition of estradiol to the medium, cells re-enter the cell cycle with the onset of S phase within 12 hours. Progression into G1 was accompanied by loss of both p21 and p27 proteins. As cells exit quiescence, both binding of cyclin D1 to cdk4, and cyclin D1 associated kinase activity rose transiently and fell by 12 hours. Activation of cyclin E/cdk2 kinase in mid-late G1 was accompanied by loss of of cyclin E-associated p21 and p27.

The requirement for p27 in the quiesence induced by estradiol depletion was investigated using antisense p27 oligonucleotides. Introduction of the antisense oligonucleotides into quiescent estradiol depleted MCF-7 cells reduced p27 levels five fold. Despite the continued absense of estradiol, the p27 antisense treated group entered into S phase, with an S phase fraction of 15 % at 34 hours post-transfection, while control cells remained arrested. P27 antisense treated cells showed a loss of cyclin E associated p27 and an increased cyclin E associated kinase activity. Thus, the antisense mediated loss of p27 was sufficient to mimic the effect of estradiol on G1-to-S phase progression in MCF-7 cells.

This data supports the notion that the loss of p27 is critical for the estradiol dependant stimulation of breast cancer cell proliferation and that an increase in p27 is required for cell cycle arrest following an inhibition of estradiol signaling.